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Precipitation of Casein from Acidic Solutions by Divalent Anions

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Abstract

Whole casein, α_s-, β-, and κ-caseins in acidic solutions are precipitated by low concentrations (about 0.005 M) of sodium sulfate. The presence of sulfate in casein solutions broadens the isoelectric precipitation zone on the acid side with no change on the alkaline side. Other divalent anions such as pyrophosphate also precipitate the caseins; chloride, phosphate, and citrate (pH 3.2) do not precipitate caseins in this concentration range. The precipitation is markedly pH-dependent; the lower the pH the greater the concentration of sodium sulfate required for precipitation. The order of precipitation (mM of sodium sulfate giving 50% precipitation at pH 3.2) for the caseins (concentration: 0.4%) is α_s (3.7), κ (4.4), whole (5.6), β (8.3). The precipitation curves are S-shaped except that for κ-casein; precipitation of this casein tends to be incomplete, even with considerable increase in the concentration of sulfate, particularly at lower pH values. Sodium sulfate does not precipitate β-lactoglobulin. The polyanions such as heparin and polyphosphates precipitate both caseins and β-lactoglobulin.

Dilute sulfuric acid has been found to be a useful means for dissolving protease and other minor components from whole casein, with solution of little or none of the caseins (6). Hydrochloric or acetic acid used at the same pH (3.5) dissolve large amounts of the caseins as well. Additional investigations have shown that these divergent results are due to the insolubility of the caseins in acidic solutions when sulfate or other divalent anions are present. The influence of pH and other factors on the precipitation of whole casein, α_s-, β-, and κ-caseins by anions such as sulfate and pyrophosphate is reported in this paper. Preliminary experiments comparing other divalent anions and polyvalent anions are briefly reported, as well as the influence of urea.

Materials and Methods

Whole casein. The casein was prepared from pooled cow's milk by precipitation with HCl at pH 4.7. It was washed several times with distilled water and dissolved and reprecipitated. The wet precipitate was extracted successively with ethanol, acetone, and ether.

α_s-Casein. This casein was prepared from either pooled milk or milk from individual cows. Some preparations were obtained by the urea NaCl-dissociation method (7) and purified by precipitation in ethanol (8). Other samples were obtained from whole α-casein prepared by urea fractionation (2) and subsequent separation of the α_s-casein by calcium chloride precipitation.

β-Casein. This casein was prepared by urea fractionation (2) and has been described in previous studies (9).

κ-Casein. This casein was prepared by the urea–sulfuric acid method (8).

β-Lactoglobulin. This protein was prepared by crystallization (4) and dried in the frozen state.

Starch gel or acrylamide gel electrophoresis was used to determine the purity of the preparations.

Sodium sulfate and sodium pyrophosphate. These were reagent-grade commercial preparations. The 0.1 M pyrophosphate solution was lowered to pH 3.2 by addition of HCl.

Preparation of casein solutions. Solutions were most readily prepared by dissolving the casein at about pH 7, then rapidly adding HCl with stirring to bring the pH to the desired value. One per cent concentrations of the caseins could readily be prepared by this procedure. The amount of sodium chloride generated, as will be shown, had negligible influence on the sodium sulfate precipitation.

Precipitation with anion. The casein solutions, usually of 5.0-ml volume, were stirred with a magnetic stirrer while the solution of anion, usually at a concentration of 0.1 M, was added. Most of the experiments were done at room temperature (about 25 C) and the mixtures permitted to stand for about 15 min before centrifuging (International Model HN)[1] for 5 min at 2,500 rpm. One-milliliter samples of the supernatant solution were removed, di-

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[1] It is not implied that the U. S. Department of Agriculture recommends the above company or its products to the exclusion of others in the same business.

luted 1:5 with water, and clarified by addition of one drop of 10 M NaOH. Protein concentrations were estimated from spectrophotometric readings at 290 mμ. All of the casein solutions except κ-casein were cloudy after centrifuging, except with excess of the sulfate anion. Presumably, these cloudy solutions represent stable micelles of casein in the presence of anion. In the experiments where the precipitation of the casein was done at various pH values with sodium sulfate present (Figure 5), half of the results were obtained by addition of increments of 0.025 M NaOH to an acidic solution (pH 2.2), others by addition of 0.025 M HCl to a solution at pH 6.7.

Results

The precipitation of whole casein by sodium sulfate at several acidic pH values is shown in Figure 1, precipitation of α_s-casein by sodium sulfate in Figure 2. Similar results were obtained with α_s-caseins made in two different ways (2, 7) and with α_s-casein from pooled milk and α_s-casein Type B. The α_s-casein Type A was somewhat less sensitive to the sodium sulfate; at pH 3.1 the concentration of sodium

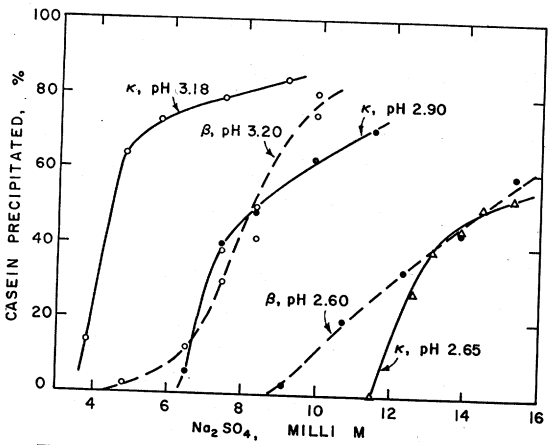

FIG. 3. Precipitation of 0.4% κ-casein and of β-casein with sodium sulfate at several pH values.

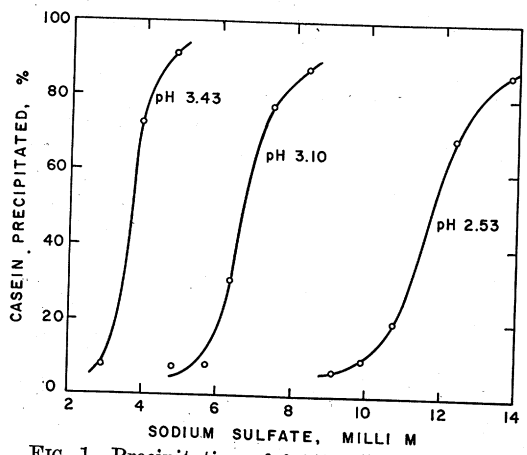

FIG. 1. Precipitation of 0.4% whole casein with sodium sulfate at several pH values.

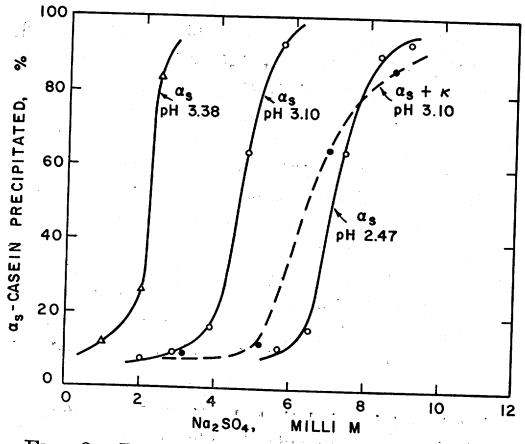

FIG. 2. Precipitation of 0.4% α_s-casein with sodium sulfate at several pH values; influence of κ-casein (1 κ to 3 α_s) on the precipitation.

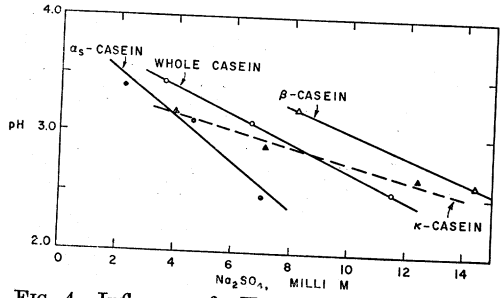

FIG. 4. Influence of pH on the precipitation of the caseins with sodium sulfate, estimated for the 50% precipitation level except for κ-casein, the 30% precipitation level.

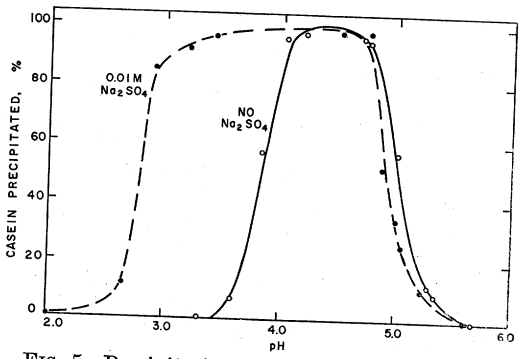

FIG. 5. Precipitation of whole casein (0.7%) at various pH values with sodium sulfate (0.010 M) and with no sodium sulfate present.

sulfate required for 50% precipitation was 6.2 mM, with a precipitation curve of the same shape as the other α_s-caseins. The influence of NaCl on the sulfate precipitation was investigated on an α_s-casein solution at pH 3.18. The presence of 0.02 M NaCl had negligible effect on the sulfate precipitation curve. NaCl at higher concentrations (about 0.08 M) will precipitate α_s-casein at this pH, even without the presence of sodium sulfate. Sodium pyrophosphate is also an effective precipitant of the caseins from acidic solutions. Sodium phosphate and sodium citrate do not precipitate the caseins from acidic solutions. κ-Casein, when added to α_s-casein, as shown in Figure 2, reduces the degree of α_s-casein precipitation by a given concentration of sodium sulfate. As shown in Figure 3 κ-casein is precipitated by sodium sulfate, but to a lesser degree than α_s-casein, and precipitation is incomplete, even with a considerable increase in the concentration of sodium sulfate. β-Casein, as shown in Figure 3, is also precipitated by sodium sulfate. The precipitation curve is similar to that of α_s-casein, but higher concentrations of sulfate are required. Results of Figure 4 summarize the influence of pH on precipitation of the caseins by sodium sulfate. In Figure 5 is shown the influence of sodium sulfate on the isoelectric precipitation of whole casein.

The effect of temperature on this precipitation was not investigated quantitatively, but it was observed that the extent of precipitation was less at a lower temperature (0 C). Small variations in temperature had negligible influence on the precipitation.

β-Lactoglobulin in acidic solutions was not precipitated by sodium sulfate. The polysulfate anions, such as heparin and chondroitin sulfate, on the other hand, precipitated β-lactoglobulin as well as α_s-casein. A polyphosphate (hexametaphosphate) also precipitated both of these proteins.

Discussion

The precipitation of the positively charged caseins by the negatively charged divalent anions, such as sulfate and pyrophosphate, most likely occurs by a binding of these ions to the caseins with a reduction in net charge. Reduction in net charge will lead to precipitation, equivalent to isoelectric precipitation, of the caseins. β-Lactoglobulin is not precipitated under these conditions, presumably because it is not precipitated in the isoelectric condition when dilute salts are present. Ability of sodium sulfate to broaden the precipitation zone of casein on the acid side with no change on the alkaline side can be attributed to the binding of sulfate anions. The negligible change on the alkaline side indicates no sulfate is bound when the net charge on the casein is negative.

The requirement for divalent ions for precipitation appears to be specific, since chloride, phosphate, and citrate anions do not precipitate the caseins under the same conditions. Phosphate and citrate, although divalent anions at higher pH values, do not provide a sufficient concentration of divalent ions at pH 3.2 to act as precipitants.

Acidic solutions of α_s-casein and β-casein form stable micelles in the presence of low concentrations of sulfate ions, perhaps analogous to the stable micelles obtained in alkaline solutions with divalent cations such as calcium ion. The κ-casein, although it does not form micelles with sulfate ion, does precipitate, although incompletely, and larger concentrations of sulfate are required than for the α_s-casein. A stabilizing action of κ-casein on α_s-casein, however, is evident from results shown in Figure 2. Larger concentrations of sulfate are required for precipitation of α_s-casein when some κ-casein is present. The mixture is precipitated as a complex, judged by the single-step shape of the precipitation curve.

The influence of the pH on precipitation as shown in Figure 4 is to be expected on the basis of charge neutralization as the explanation of the precipitation. The greater net positive charge at the lower pH values requires larger concentrations of sulfate for neutralization and consequent precipitation. The precipitation-pH curves for whole, α_s-, and β-caseins extrapolate to pH 3.9 for zero concentration of sulfate. This is close to the pH of 3.85, giving 50% precipitation when no sodium sulfate is present (Figure 5).

Polyvalent anions such as heparin, chondroitin sulfate, and polyphosphates precipitate both casein and β-lactoglobulin. Probably these and other polyvalent anions are general precipitants of positively charged proteins. The ability of metaphosphates to precipitate proteins from acidic solutions has been known for a long time (1, 5) and a correlation demonstrated between the metaphosphate bound and the number of positively charged groups of the precipitated proteins (5). It appears from the comparative results with divalent anions and polyvalent anions that in the latter case cross-bonding occurs to a degree such that large, insoluble aggregates are formed.

The precipitation of proteins by divalent anions may be useful for fractionation when used under conditions where protein complexes

are dissociated. The urea–sulfuric acid method for preparing κ-casein (8) is probably of this type. In this method acid-precipitated whole casein is dissolved in 6.6 M urea, and sulfuric acid added to a concentration of about 1.2 N (pH about 1.5). Under these conditions the α_s- and β-caseins are preferentially precipitated and κ-casein of quite high purity remains in solution. Under conditions used in the present experiments (pH 3.2) whole casein with 2.0 M urea present required 12 mM sodium sulfate for the appearance of a precipitate and even with 20 mM concentration only 50% of the casein had precipitated. The concentration of urea, however, was probably not sufficient to dissociate the casein complex, for both the precipitate and the soluble fraction appeared to be identical on gel electrophoresis. Thus, precipitation in this system appears to be incomplete, similar to that shown by κ-casein even without urea. Use of a polyanion, a polyphosphate, in urea–acetic acid has provided a successful procedure for the isolation of α_s-casein (3). In this case the α_s-casein is preferentially precipitated, whereas the β- and κ-caseins remain in solution.

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